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Specific Antigen Transgenic Mice

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## Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5-11
Key Research Accomplishments.....	12
Reportable Outcomes.....	13-14
Conclusions.....	15-16
References.....	17
Appendix.....	18

## Introduction

In Western society, prostate cancer represents the most common non-cutaneous cancer in men and is the second leading cancer related cause of death. The widespread occurrence of the cancer enables it to account for almost forty thousand deaths a year in the U.S. despite the fact that the majority of individuals who contract the disease die of other causes. Early disease can be cured via surgical resection or three dimensional external beam radiation but is frequently avoided since the slow growth of the tumor generally allows the person to die of their other comorbidities without the side effects of the definitive treatments. Unfortunately, if metastatic disease occurs, therapeutic regimens are only capable of delaying the tumor's growth or providing palliation and the person will probably succumb to prostate cancer. Our work attempted to develop an anti-prostate cancer vaccine that specifically induces an immune response against a protein expressed by prostate cancer cells, prostate specific antigen (PSA). We focused on demonstrating the ability of a vaccine to induce an anti-tumor response targeting PSA in a setting where PSA is considered a self antigen and forces of tolerance could confound the vaccine's efficacy. Given the ultimate goal of applying this vaccine to the clinical situation, experiments were also performed to develop a vaccine for human application using components restricted to one haplotype of the human class I MHC system, HLA-A\*0201 (A2). The previous annual reports detailed our success in both targeting the self-antigen PSA in PSA transgenic mice and identifying and using A2 restricted epitopes of the PSA protein in vaccines that protected transgenic mice containing a quasi-human immune system from PSA expressing tumor cells. These results allow us to continue to develop the vaccine to target PSA that may ultimately lead to phase I clinical trials for use in patients suffering from prostate cancer. Additionally, successful establishment of the strategies and techniques necessary for creating a vaccine targeting the PSA protein of prostate cancer may eventually lead to the ability to treat other cancers if their tumor associated antigens were incorporated into a similar vaccine.

## Body

The following report represents the work completed at the end of the funding period for the grant entitled: Prostate Cancer Immunotherapy Development in Prostate Specific Antigen Transgenic Mice (DAMD17-98-1-8480). The grant was divided into three technical objectives designed to initiate the development of an anti-prostate cancer vaccine targeting the human prostate specific antigen gene (PSA) expressed by 95% of the prostatic adenocarcinomas. The first objective was intended to provide proof of the possibility to target the PSA protein of tumors in transgenic mice who express PSA in their prostates due to the germline inclusion of the PSA gene fused to the prostate specific, rat probasin promoter. The second objective was designed to identify PSA epitopes restricted to the human HLA-A\*0201 haplotype and immunogenic in the A\*0201/Dd (A2Dd) transgenic mice that have been engineered to contain a quasi-human immune system. The third objective was designed to evaluate the ability of the defined PSA epitopes to induce protective and therapeutic immunity with the ultimate goal of creating an optimized vaccine capable of eradicating preexisting tumors in F1 hybrid mice containing both the PSA and A2Dd transgenes. The successful completion of these experiments would provide promising preliminary data for the eventual translation of the work into phase I clinical trials.

As detailed in the previous two annual reports, technical objectives one and two were completed. The identification of five immunogenic PSA epitopes and demonstration of their ability to provide protective anti-PSA immunity in A2Dd mice when used in a dendritic cell (DC) based vaccine was also previously described as completed parts of technical objective three. The disastrous blight of the murine hepatitis virus (MHV) that decimated both transgenic mice strains used in this work halted our research as mentioned in the previous report. Our Department of Comparative Medicine initially decided to attempt to eliminate the virus utilizing 'burn out' and rederivation methods.

Although originally told to expect a delay of six months until the mice could be utilized, the actual time of recovery took two years. Initial plans to simultaneously use both a 'burn-out' period method and a rederivation of the colony to insure a rapid recovery in case one technique failed were abandoned by our Department of Comparative Medicine due to the cost of the rederivation process. Unfortunately, six months after the initiation of the 'burn-out' process, both strains of transgenic mice were still found to be infected and capable of spreading the virus and the mice were sent to a mouse supply company for rederivation. Clean, MHV-free mice were finally returned to the laboratory in December of 2001 and breeding efforts have been focused on increasing the colony size to maintain an adequate number of mice to provide the numbers of mice necessary for experimentation.

While awaiting the return of the transgenic mice, laboratory personnel pursued projects that did not require either mouse strain. As will be described in detail below, work was concentrated in three distinct areas. First, the identification of the five relevant PSA peptides and proof of their ability to induce anti-tumor immunity allows their inclusion in a prostate cancer vaccine. Parallel work in the lab revealed the impressive strength of a vaccine strategy utilizing a DNA construct engineered to contain the corresponding nucleic acid sequence of each peptide in a 'beads on a string' fashion.

Using the five relevant epitopes, a similar DNA vaccine has been created and is currently being tested for its ability to induce peptide specific immunity. Second, utilizing a transgenic mouse model, TRAMP, that contracts *de novo* prostate cancer due to the prostate specific expression of the SV40 large and small T antigens, four prostate specific genes were identified and found to be homologs of the human prostate genes. Finally, measurement of the *de novo* arising tumors in the TRAMP model is difficult since they develop deep within the perineum. We have tentatively developed a blood test measuring serum levels of PSA in F1 hybrids of PSA x TRAMP transgenic mice that may correspond to tumor burden.

With the long awaited return of the transgenic mice, the experiments originally proposed in the grant and begun before the MHV outbreak will now continue anew. As will be discussed below, the remaining tasks of technical objective three may now be completed aided by the work done while pending the return of the transgenic mice. Successful completion of these experiments will allow the development of the vaccine to continue and ultimately reach clinical trials.

#### **Technical Objective One**

Technical objective one was divided into three separate tasks to determine if it is possible to target the PSA protein in a setting where it is considered a self antigen. The three tasks involved 1) the creation of the model, 2) vaccination of the PSA transgenic mice with either PSA DNA or whole protein emulsified in adjuvant with subsequent challenge with PSA expressing, syngeneic tumor cells and 3) the measurement of the efficacy of any induced immunity. As previously reported, the proposed vaccination strategies failed and thus other immunization methods were utilized. We found that vaccination of PSA transgenic mice with the human prostate cancer cell line, LNCaP, that greatly overexpresses PSA was able to elicit an anti-tumor response that was PSA specific. This demonstrated the ability of a vaccine to break any potential tolerance that existed in the PSA transgenic mouse to its self antigen, PSA, and induce a protective anti-tumor response. This enabled our work to continue since an immunotherapy targeting PSA as a self antigen was no longer just a theoretical possibility.

In the previous report, we detailed the creation of an alternate cell line superior to the one used in the above experiments since it did not have the characteristic of being a regressor line. This cell line was found to curiously only express the message for PSA and not the protein. Although we stated we would attempt to retransfect this cell line with the PSA gene, we have decided against this course of action since our work has developed to a point where our experiments with PSA transgenic mice are performed in F1 hybrids with the HLA-A\*0201/Dd (A2Dd) or TRAMP mice whose resultant H-2<sup>b/q</sup> haplotype renders a tumor line of solely the H-2<sup>q</sup> haplotype useless.

#### **Technical Objective Two**

Technical objective two was also divided into three tasks whose purpose was to identify the HLA-A\*0201 (A2) restricted PSA epitopes and determine their immunogenicity in the A2Dd transgenic mouse. Task one served to create minigene DNA constructs for each of nine candidate PSA peptides selected through computer modeling and competition assays. Task 2 involved the vaccination of A2Dd mice with either the DNA minigenes or the individual peptides emulsified in adjuvant to determine

in task 3 which method provided the induction of the strongest peptide specific immunity in standard chromium release assays.

As reported in the first two annual reports, we altered our vaccination strategy to incorporate the peptides into a dendritic cell (DC) based vaccine to take advantage of the powerful ability of DCs to induce cytotoxic T lymphocyte (CTL) responses. We identified four peptides of the original nine that were able to induce a peptide specific CTL response, PSA-1, PSA-2, PSA-3 and PSA-7. Although its immunogenicity was never proven, we added PSA-6 to the list of peptides to continue working with due to the fact that it was actually PSA-1 plus one amino acid. Of these five peptides, binding assays for the murine MHC molecules revealed that only PSA-2 cross reacted with one of the mouse MHC molecules. The identification of these peptides completed this technical objective.

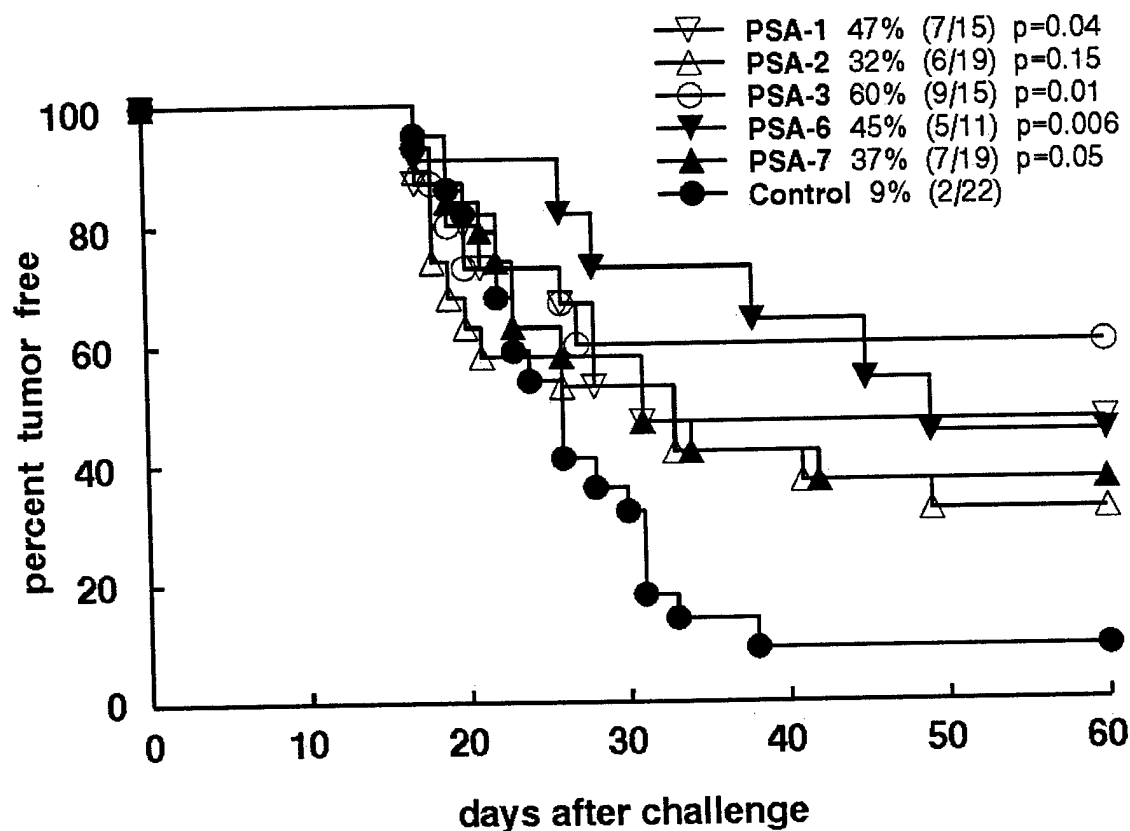
### Technical Objective Three

This technical objective was separated into five tasks intended to combine the results from the first two objectives and create an optimal vaccine for the anti-prostate cancer immunotherapy targeting PSA. Task 1 recounted the need to create two PSA expressing tumor cell lines for use in the A2Dd (H-2<sup>b</sup>) and the F1 hybrids of the PSA x A2Dd transgenic mice (H-2<sup>b/q</sup>). Additionally, this initial task called for the creation of the optimal vaccine DNA minigene construct if the DNA strategy was found to be the dominant immunization method. Task 2 then served to test the ability of the optimal vaccine in both the A2Dd mouse and PSA x A2Dd F1 hybrid mouse where issues of tolerance may confound the vaccine's efficacy. Tasks 3, 4 and 5 detailed the experiments necessary to evaluate the memory response, therapeutic ability and chemotherapeutic augmentation of any therapeutic ability, respectively.

As described in the previous reports, we altered our strategy since our vaccination methodology in technical objective two used a DC based approach instead of a DNA or free peptide approach. Since we felt it more important to evaluate each immunogenic peptide's ability to induce effective anti-tumor immunity, each peptide was evaluated individually. **Figure 1** contains the cumulative protection data for each individual peptide as previously reported in table form in the last annual report. We concluded that all five peptides were capable of inducing the desired response and deserving of inclusion in an optimal vaccine despite the fact that PSA-2 did not reach statistical significance using the logrank test for the modified Kaplan-Meier curves.

As mentioned above, parallel work in our laboratory revealed the necessary components of a DNA construct containing the nucleic acid sequence of each peptide arranged in a 'bead on a string' approach. As shown in Velders *et al.* 2001, the inclusion of a T helper epitope, ubiquitination sequence and presence of three amino acid spacers between each epitope with a characteristic alanine-alanine-tyrosine sequence induced a strong CTL response capable of eradicating preexisting tumors. We constructed a similar construct in the pCDNA3 vector containing all five epitopes and the HBV core antigen (HBVcAg) T helper epitope (128-140, TPPAYRPPNAPIL) pictured in **Figure 2**. It has been sequenced and found to be accurate and is currently being tested for its ability to induce peptide specific CTL immunity in the now returned A2Dd transgenic mice.

For the other part of task 1, the creation of a tumor cell line for use in the F1 hybrids of a PSA x A2Dd mouse (H-2<sup>b/q</sup>), we decided to alter our approach. We



**Figure 1.** Modified Kaplan-Meier curves depicting the cumulative data from tumor protection experiments where A2Dd transgenic mice were vaccinated with DCs pulsed with individual peptides and subsequently challenged with PSA/EL4A2Kb. Control animals were vaccinated with either the HPV 16 E7 86-93 peptide and HBVcAg helper peptide or the helper peptide alone. In the legend, the numbers after each peptide represent the percent tumor free at 60 days, actual numbers of tumor free mice/number of mice used and corresponding p value obtained via the logrank test, respectively. A statistically significant difference in the survival curves exists for all PSA peptides except PSA-2.



originally planned to create this cell line through transformation of primary prostate epithelial cells of a hybrid mouse or transfection of an established tumor cell line with the missing class I MHC and PSA gene. Currently, we are using the fact that the TRAMP model spontaneously develops prostate cancer and previous work has developed tumor cell lines from the resection and culture of these transformed prostate cells. We are attempting to breed a triple transgenic mouse that expresses transgenes from the PSA, A2Dd and TRAMP mice for the purpose of allowing a male to develop prostate cancer to harvest the transformed cells. Creation of this cell line would complete task 1.



**Figure 2.** DNA map of the minigene construct containing all five relevant PSA peptides with spacers (AAY) and containing the necessary T helper epitope (HBVc) and ubiquitination sequence (Ub). This construct was spliced into the pCDNA3 plasmid.

Experiments to satisfy the proposed work in task 2 are also commencing now that the A2Dd and PSA mice are returning. Once we have completed testing the minigene construct for its ability to induce a CTL response, we will conduct experiments to see if it can protect A2Dd mice from a challenge of the same tumor cell line used in the experiments graphed in figure 1, PSA/EL4A2Kb. We believe that the construct should provide better protection for the mice than what was seen in figure 1 for two reasons. One, the inclusion of multiple epitopes should induce more powerful immunity since CTLs specific for multiple epitopes should be induced. Second, the inclusion of multiple epitopes disallows the tumor cells from escaping the immune reaction via mutation of the targeted epitope.

With the completion of the second part of task 1, the creation of the PSA and A2Dd expressing tumor cell line syngeneic for the F1 hybrid mice of a PSA x A2Dd cross, we will conduct parallel experiments in the F1 hybrids. These experiments will allow us to determine if any tumor protection elicited in the A2Dd mouse can continue to provide anti-tumor immunity when the targeted antigen is a self antigen and the forces of tolerance may interfere. This mouse model will finally allow for the completion of task 2 with the evaluation of the PSA expressing normal prostate cells after vaccination for determination of the presence of autoimmunity.

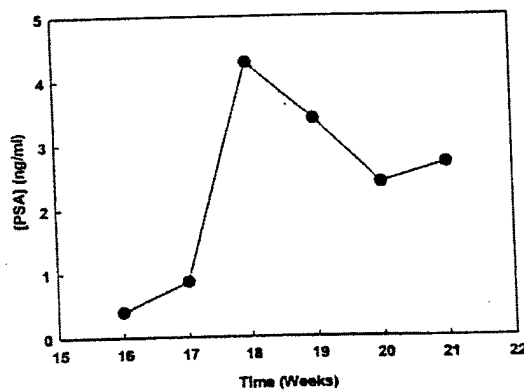
Tasks 3, 4 and 5, the evaluation of a memory response, therapeutic ability and chemotherapeutic ability, respectively, will be completed if the optimal vaccine has been shown to induce a protective anti-PSA response in the A2Dd or PSA/A2Dd F1 hybrid mice. Completion of these tasks as planned will conclude the experiments proposed in the original grant. The unfortunate MHV outbreak and prolonged recovery of usable, clean mice has preempted our ability to finish our work in the time we originally determined to need.

The results we have collected to date have spawned interesting projects associated with the original proposal and have come to the forefront of our efforts due to the long delay in recovering usable transgenic mice. We were able to obtain and use the TRAMP

mice while waiting for our transgenics to return since they happened to be housed at facilities not affected by MHV. Three areas of research using the TRAMP mice are currently demanding time within the laboratory and will possibly serve to augment the work done to date and produce a higher quality vaccine with a greater chance of success in the clinical situation.

First, evidence exists in the literature to support the notion that a subcutaneously injected tumor cell method for mimicking cancer may not be an appropriate model. The demonstration of the ability of anti-tumor immunity to eradicate a subcutaneously injected tumor cell line without affecting *de novo* arising tumors containing the same targeted antigen in the same mouse indicates the need to create a cancer model that more closely resemble the human condition. We believe that the use of the TRAMP mouse to create a triple transgenic animal expressing the PSA, A2Dd and TRAMP models will allow us to more accurately assess the strength of our optimal vaccine. Currently, the returned transgenic mice are being continuously backcrossed into mice containing at least two of the transgenes in order to eventually develop a stable triple transgenic mouse strain.

One caveat with this mouse model is the inability to accurately measure the tumor burden of the mice since the tumors arise within the prostate located deep within the perineum. A second line of research pursued in the lab has been to develop a method of accurately measuring the tumors of these mice. With the return of the PSA transgenic transgenics have been followed for tumor development. Using a sensitive ELISA assay, measurement of serum PSA levels have been found to rise in the mice corresponding to the characteristic timing for the establishment of tumors in the mice (see Figure 3).



**Figure 3.** Measurement of serum levels of PSA in PSA x TRAMP F1 hybrids during development of spontaneous tumors. TRAMP mice characteristically develop tumors beginning around 16 weeks.

Further testing needs to occur to ensure the accuracy of the data and to provide a 'standard curve' to correlate PSA level with tumor burden. Perfection of this method is considered crucial to continuing to use the TRAMP model for evaluation of the optimal vaccine.

One final area of research involved the search for alternate tumor antigens within the prostates of the TRAMP mice. In humans, a multitude

of prostate specific antigens exist creating a plethora of potential targets to potentially

include in an anti-prostate cancer vaccine. Since one method of tumor escape has been documented to involve the loss of the entire targeted antigen, it is logical to include peptides from multiple antigens to help prevent the tumor from evading immune responses. While waiting for our transgenic mice to return, we evaluated transformed prostate cells from TRAMP mice and discovered four prostate antigens. Use of a computer to search known databases for identification of the discovered transcripts revealed the four proteins to be the mouse homologs of human proteins expressed by the prostate including, STEAP, PSCA, mesothelin and PSMA. Mesothelin was not previously described to be expressed in prostate cancer but our studies show that it is a candidate antigen. We plan on identifying relevant epitopes from these antigens to strengthen our optimal vaccine in terms of broadening the scope of antigens it targets.

The information contained within this report details the results we have collected during the funding period for this grant. The unfortunate MHV outbreak and prolonged recovery time for the return of clean mice greatly hampered our efforts. We were successful in providing proof that it is possible for an immunotherapy to target the PSA protein in a setting where it is considered a self antigen and issues of tolerance may interfere with the vaccine's efficacy. Additionally, we were able to identify five relevant PSA peptides restricted to the A2 haplotype and demonstrated their ability to protect mice from a challenge of PSA expressing, syngeneic tumor cells. With the exception of PSA-2 that may have exerted its effects through either the human or mouse MHC molecule, the other four peptides provided their anti-tumor immunity dependent completely on human components. These results demand their inclusion in an optimized vaccine that we have now produced through the creation of a minigene DNA construct where the PSA epitopes are arranged in a 'beads on a string' approach. Now that the transgenic mice have returned, we are poised to perform critical experiments whose results will help shape the strategy and components of an anti-prostate cancer vaccine ultimately slated for use in a clinical setting.

### **Key Research Accomplishments**

1. Creation of a mini-gene construct incorporating each PSA peptide restricted to the HLA-A\*0201 haplotype and effective at protecting HLA-A\*0201/D<sup>d</sup> transgenic mice in a design previously shown to induce strong protective and therapeutic anti-tumor immunity.
2. Creation of a method potentially capable of determining tumor size in a situation where usual techniques for measurement fail due to the location of the tumors deep within the perineum.
3. Identification of murine homologs of human prostate antigens, STEAP, PSCA, mesothelin and PSMA, in prostates of TRAMP transgenic mice which may serve as additional targets for an anti-prostate cancer vaccine.

## **Reportable Outcomes**

### Manuscripts, abstracts, presentations and grants:

- Yang D, Markiewicz MA, Qin QZ, Le Poole IC, Kwon ED and Kast WM. Gene expression analysis reveals mesothelin present in prostate cancer. Submitted for publication, 2002.
- Holt GE, Jay G, and Kast WM. Vaccination of PSA transgenic mice with the PSA positive human prostate cancer cell line, LNCaP, induces a tumor protective anti-PSA response. To be submitted for publication 2002.
- Holt GE, Celis E, and Kast WM. Induction of protective anti-tumor immunity in HLA-A\*0201/Dd transgenic mice via vaccination with A\*0201 restricted PSA peptide pulsed dendritic cells. To be submitted for publication, 2002.

### Patents and licenses applied for and/or issued:

None

### Degrees obtained that are supported by this award:

- Holt, GE, Doctor of Philosophy in Pharmacology and Experimental Therapeutics as part of a combined M.D./Ph.D. program at Loyola University Chicago. June 2002.

### Development of cell lines, tissue, or serum repositories:

None

### Informatics such as databases and animal models, etc:

Gene registrations in GENBANK and EMBL databases

- Mouse Six-Transmembrane Epithelial Antigen of the Prostate (mSTEAP), Accession Number AF297098. Yang D, Velders MP, Kast WM, August 2000.
- Mouse Prostate Stem Cell Antigen (mPSCA), Accession Number AF319173. Yang D, Holt GE, Kast WM, November 2000

### Funding applied for based on work supported by this award:

- Applied for an NIH grant (RO1 CA099463) entitled Cancer Therapy-Related Use of Genetically Engineered Mice for the period of 1/03 to 12/07.

Employment or research opportunities applied for and/or received on experiences/training by this award:

- Three year fellowship (8/02-7/05) from the Damon Runyon Cancer Research Foundation. Project title: Cancer Prevention and Immunotherapy in a Clinically Relevant Chronic Prostate Cancer Model (DRG 1727-02, Markiewicz). \$128,500 (total direct cost). Accepted.
- Two year fellowship (2002-2004) from AFUD. Project Title: Cancer Prevention and Immunotherapy in a Clinically Relevant Chronic Prostate Cancer Model (Markiewicz). \$60,000 (total direct cost). Declined
- Three year residency (6/02 -6/05) in Internal Medicine at Georgetown University (Holt).

## Conclusions

The purpose of this grant was to initiate the development of a prostate cancer immunotherapy to specifically target the expressed PSA protein of the prostatic adenocarcinomas. Using a PSA transgenic model to simulate the human condition where PSA is considered a self-antigen, we demonstrated the ability of a vaccine to induce an anti-tumor response specifically targeting PSA in a situation where issues of tolerance could interfere. To begin to translate the work into a human context and use a peptide focused approach, we used the A2Dd transgenic mouse containing a quasi-human immune system to identify five PSA peptides restricted to the human A2 haplotype capable of inducing a protective anti-tumor immunity. These five peptides have subsequently been included in a DNA minigene construct using a similar design to a DNA vaccine strategy proven to be quite effective in eradicating preexisting tumors in mice in our laboratory. Creation of this optimal vaccine will now allow us to evaluate it for its ability to protect mice from tumor challenges, induce a potent memory response and eradicate preexisting tumors with the possible aid of chemotherapeutic augmentation.

Our work was halted due to the outbreak of the murine hepatitis virus that afflicted our transgenic mice. The failure of the 'burn out' method to provide virus free mice coupled to the lengthy rederivation process that eventually did produce clean mice caused a delay of two years. With the return of the transgenic mice, recent breeding efforts have been focused on repopulating the colony to produce the numbers of mice needed for experimentation.

While awaiting the return of usable mice, the laboratory created and began testing the so called optimal vaccine. Upon demonstration of its ability to induce peptide specific CTL immunity, the experiments of tasks two through six of technical objective three will be conducted. The successful completion of these experiments should provide results capable of translating this approach to the human situation for eventual inclusion in phase I clinical trials.

This waiting period also produced two other lines of research utilizing the TRAMP transgenic mouse model that spontaneously contracts prostate cancer. First, subtraction hybridization of RNA from the transformed prostate cells of TRAMP mice allowed the identification of four prostate proteins that were found to be homologs of human prostate proteins, STEAP, PSCA, mesothelin and PSMA. These proteins can now be analyzed for candidate A2 binding epitopes for eventual incorporation into a final vaccine to both increase the vaccine's strength and broaden its scope of targeted antigens to prevent putative tumor escape mechanisms.

Secondly, the plan to eventually utilize the TRAMP model as the source of a more realistic tumor model in the experiments was hindered by the inability to accurately measure tumor volume due to the growth of the tumors deep in the perineum. We have obtained preliminary evidence for the correlation of the level of circulating PSA and tumor burden in the PSA x TRAMP F1 hybrids would potentially alleviate this dilemma. Further testing will hopefully confirm this early data and provide a sense of tumor size for a given serum PSA level.

With the return of the clean transgenic mice, this laboratory has resumed its work towards the development of a prostate cancer vaccine targeting the PSA protein. With the new developments, our focus concerns the use of the DNA minigene construct as the

optimal vaccine and are currently testing its efficacy. We plan to broaden our efforts to include the additional antigens discovered in the TRAMP mouse's prostate and will hopefully be able to utilize the developed blood test as a surrogate marker of tumor size as we incorporate the TRAMP model as a more realistic source of tumors in our experiments. Through these experiments, the realization of our ultimate goal of an effective anti-prostate cancer vaccine becomes closer.



## References

Velders MP, Weijzen S, Eiben GL, Elmishad AG, Kloetzel PM, Higgins T, Ciccarelli RB, Evans M, Man S, Smith L, Kast WM, Defined flanking spacers and enhanced proteolysis is essential for eradication of established tumors by an epitope string DNA vaccine, *J Immunol* 166(9):5366-5373, 2001.